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Simple, Rapid, and Quantitative Release of Periplasmic Proteins by Chloroform

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We introduce a method by which periplasmic proteins can be released rapidly, simply, and quantitatively by treating cells with chloroform. All the amino acid-binding proteins tested maintained their activity during chloroform treatment. This method makes practical the analysis of the periplasmic protein complement of a large number of strains.

The periplasmic proteins of gram-negative bacteria are defined operationally as those proteins which are released into the medium by mild osmotic shock (2). These proteins comprise ca. 10 to 15% of the total cell protein and include numerous transport-related binding proteins and a variety of enzymes, which appear to lie outside the cytoplasmic membrane layer of the cell envelope (2). The cell compartment in which these proteins are located is called the periplasm, and it constitutes a sizable fraction of the total cell volume (14). Studies involving periplasmic proteins often require that they be initially separated from the bulk of the cell protein by osmotic shock. This is accomplished by first suspending washed cells in a concentrated sucrose solution in the presence of EDTA and then subjecting them to osmotic shock in cold distilled water. The procedure is cumbersome because it requires a number of centrifugation steps. Therefore, it has been difficult to use this procedure to screen large numbers of strains rapidly for the presence or absence, nature (such as altered mobility), and activity of periplasmic proteins (see, e.g., references 5 and 6). In some cases, the protein of interest may be visible in a sodium dodecyl sulfate gel electrophoretogram of intact cells (e.g., if its size is such that it moves to a relatively uncrowded region of the gel and if it is sufficiently abundant). However, the protein of interest often cannot be seen. In such instances, simply separating the periplasmic proteins from the rest of the cellular protein achieves an approximate 10-fold purification. The use of a rapid cell permeabilization procedure (10) allows the activity of certain periplasmic proteins to be assayed. However, this procedure is not useful if the only measurable activity of the periplasmic protein of interest is substrate binding, nor is it useful for ascertaining the presence of inactive proteins or determining the specific localization in the periplasm of either active or inactive proteins. Thus, the separation of periplasmic proteins from the bulk of the cell protein is a very important step in characterizing many mutations which affect periplasmic proteins and mutants which may have been collected by a variety of selection methods.

We describe here a method for specifically releasing periplasmic proteins rapidly, simply, and reproducibly by exposing cells to chloroform. (We refer to chloroform shock and chloroform shock fluid in this study, although we assume that the proteins released by treating cells with chloroform are liberated by a process unrelated to osmotic shock.) In our standard procedure, 2-ml bacterial cultures are grown to saturation overnight in culture tubes (13 by 100 mm). The cells are collected by centrifugation for 10 min at 1,100 × *g*. The use of rubber centrifuge adapters allows centrifugation of the culture tubes directly, without breakage. The supernatant is decanted thoroughly. When many tubes are handled simultaneously, it is necessary to decant them rapidly to avoid resuspension of the cells. The cell pellet is resuspended by brief vortexing in the residual medium, and 20 μl of $CHCl_3$ is then added. After brief vortexing, the tubes are maintained at room temperature for about 15 min, and then 0.2 ml of 0.01 M Tris hydrochloride (pH 8.0) is added. The cells are separated by centrifugation at 6,000 × *g* for 20 min, and the supernatant fraction containing the periplasmic proteins is withdrawn with a Pasteur pipette. It is necessary to obtain a tightly packed cell pellet at this stage because this is the final supernatant, which should be as free as possible from contamination by cells. It is advisable to withdraw only a portion of the supernatant. We found that it is possible to centrifuge disposable culture tubes at 7,000 rpm in an SS34 Sorvall rotor without incurring any breakage if a no. 366 rubber Sorvall adapter is used. Thus, the entire operation is carried out without any transfer of the cultures. Because the operations required are so simple, the only limit is dictated by the number of tubes that can be centrifuged at one time. The no. 369 Sorvall adapter within a no. 456 adapter allows centrifugation of 30 samples in the GSA rotor simultaneously.

Figure 1 shows an acrylamide gel electrophoretogram of the proteins liberated by this method, by a few variations of this technique, and by the standard osmotic shock procedure. The patterns of bands obtained by chloroform shock and osmotic shock are very similar (Fig. 1, lanes 5, 6, and 8), and the time of incubation with chloroform has little effect (compare lanes 5 and 6). It is interesting that toluene, which is often used in cell permeabilization procedures, not only fails to improve protein release, but actually interferes with it (compare lanes 1, 2, 3, and 7 with lanes 5 and 6). A mixture of chloroform and toluene has been used to release periplasmic proteins from colonies on a petri plate (3), but no optimization of the procedure was presented in that study. Chloroform shock releases the major periplasmic components as effectively as osmotic shock does (Fig. 1). To identify other solvents which would also release periplasmic proteins, we replaced the chloroform with phenethyl alcohol, ether, ether-chloroform (1:1), or dimethyl sulfoxide, keeping other conditions essentially the same, except that

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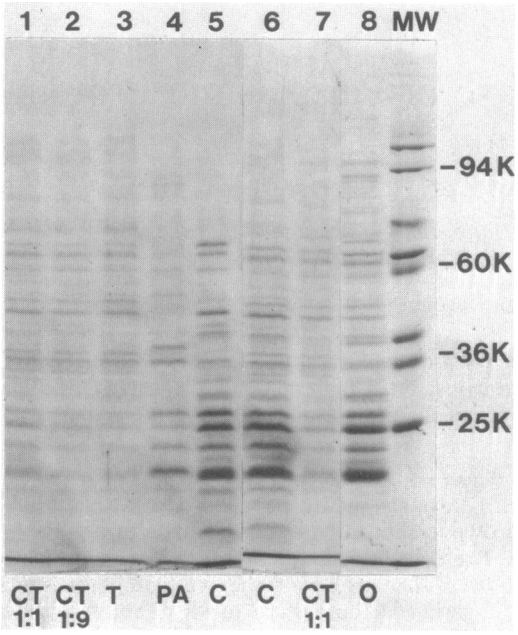

FIG. 1. Electrophoretogram of periplasmic proteins released from cells of strain TA831 (*ΔhisF645*) by various treatments. Lanes: 1, chloroform-toluene (1:1); 2, chloroform-toluene (1:9); 3, toluene; 4, phenethyl alcohol; 5 and 6, chloroform; 7, chloroform-toluene (1:1); 8, osmotic shock fluid; MW, molecular weight standards (94K = 94,000 molecular weight). Lanes 1 to 5, 20 μl of solvent(s) added and standard procedure used, except for the sample in lane 4, which was incubated at 37°C for 15 min before buffer was added. Lanes 6 and 7, solvent (20 μl) was added immediately before 200 μl of 0.01 M Trishydrochloride (pH 8); incubation was for 30 min. The strain was cultured to saturation in minimal salts medium with 0.4% glucose as the carbon source (12). Shock fluids were diluted twofold with twice-concentrated Laemmli sample buffer (8) and boiled for 2 min, and 15 μl was electrophoresed on 10% acrylamide gels as described previously (1). Gels were stained with Coomassie blue.

phenethyl alcohol-treated cells were incubated for 15 min at 37°C before addition of the buffer (Fig. 1, lane 4). All of these treatments caused release of lesser amounts of proteins and so were not investigated further (data not shown). Adding 0.01 M $MgCl_2$ to the cells before chloroform treatment decreased the amount of protein released, but the pattern of proteins was qualitatively the same (data not shown). The order in which the chloroform and the buffer are added seems to have little effect on the nature and amount of proteins released. However, adding the buffer a short time after the chloroform gives more reproducible results; perhaps this sequence allows some of the excess chloroform to evaporate and thus prevents it from interfering with the tight packing of the cell pellet. The amount of chloroform added is also important for the same reason. Amounts greater than 20 μl interfere with cell packing and should be avoided.

Many periplasmic proteins are found to be unusually stable to heat and proteolytic digestion; they may also be particularly resistant to chloroform. We found this to be true for all of the periplasmic binding proteins we assayed. Thus, these proteins can be readily assayed for activity in a chloroform shock fluid. The amounts of several different periplasmic binding proteins (i.e., the glutamine-binding protein, the lysine-arginine-ornithine-binding protein, and other arginine-binding proteins) released were essentially the same with both chloroform and osmotic shock, as shown in Table 1 for two different strains. Similar results were obtained for the histidine-binding protein (data not shown). The total amount of protein released by the two procedures was also comparable (ca. 16% of the total cell protein). Perhaps some periplasmic proteins cannot withstand exposure to chloroform, and so each of these proteins will have to be tested individually. When the released periplasmic proteins only need to be examined by sodium dodecyl sulfate gel electrophoresis or immunological techniques (6), such inactivation would be irrelevant. To demonstrate that chloroform released mainly periplasmic proteins and left the inner membrane essentially impermeable to cytoplasmic proteins, we assayed the chloroform shock fluid for the presence of cytoplasmic enzymes. Glutamine synthetase, which is not inactivated by chloroform, was present in very small, comparable amounts in chloroform and osmotic shock fluids (Table 1). In a strain grown under derepressing conditions, only about 1% of the total glutamine synthetase activity appeared in either shock fluid. Similar results were obtained with β-galactosidase (10) in strain TA2365 (*pho-25*), which carries an F′ *lac*$^+$ *phoA*$^+$ plasmid (data not shown).

To determine whether the chloroform shock procedure would be effective on larger volumes of cells, we treated 50-ml cultures in an identical, scaled-up way and obtained identical results by one-dimensional gel electrophoresis (data not shown). A more detailed comparison of the proteins released by the large-scale chloroform shock method with those released by the osmotic shock method was done by two-dimensional gel electrophoresis (Fig. 2). The two procedures gave similar patterns for most of the abundant proteins. The chloroform shock fluid had a background of many minor spots, which may represent either a small amount of cytoplasmic proteins being released or minor

TABLE 1. Proteins released into shock fluids

Strain[a]	Shock treatment	Binding (pmol of substrate/mg [dry wt] of cells) of[b]:			Glutamine synthetase (μmol/10 ml)[c]	Total protein (mg)
		Gln	Lys-Arg-Orn	Arg		
TA831	Chloroform	11.9	20.7	16.5	0.4	3.7
	Osmotic	7.5	20.3	15.8	0.6	4.0
SK97	Chloroform	0.7	<2	10.7	<0.2	2.7
	Osmotic	0.6	<2	10.8	<0.2	3.0

[a] Cells were grown under nitrogen-limiting conditions (4). Strain SK97 carries *ΔhisF645 ntrC73*. This strain cannot increase the level of glutamine synthetase or of the glutamine-binding or lysine-arginine-ornithine-binding protein under nitrogen limitation (15).

[b] See reference 9 for methods and calculations.

[c] Units represent total glutamine synthetase in the chloroform and osmotic shock fluids from 100 ml of cells (7, 13). In strain TA831 this activity amounted to about 1% of the total glutamine synthetase since the activity in crude extracts, prepared by disrupting an equivalent number of cells at 18,000 lb/in^2 in a French pressure cell, was ~46 U. The activity was unaffected by the addition of chloroform to either the crude extract or the shock fluid.

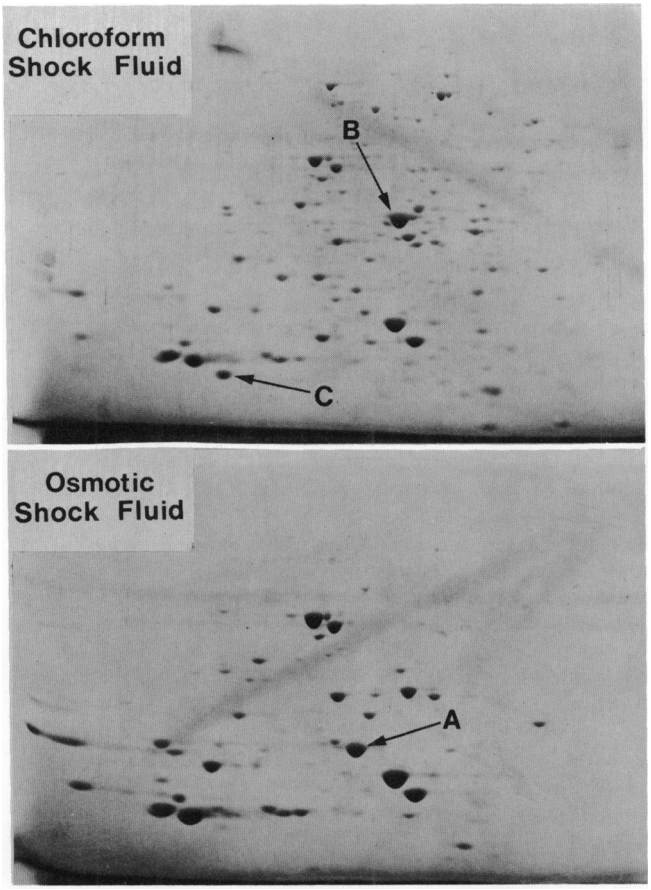

FIG. 2. Two-dimensional gel electrophoretogram of shock fluid from strain TA3292 (*dhuA1 ntrB139*); the latter mutation increases the level of several periplasmic proteins. The chloroform (top) and osmotic (bottom) shock fluids obtained from 50 ml of cells were concentrated 10-fold by lyophilization, and 10 μl, containing 45 μg of protein, was analyzed by two-dimensional gel electrophoresis (11). The basic end is on the left. Most of the abundant proteins were present in equal amounts in both shock fluids, with the exceptions of a protein released more extensively by osmotic shock (arrow A) and two proteins released more extensively by chloroform shock (arrows B and C). We do not know the nature of these proteins.

contamination by intact cells. Because the purpose of this method is to yield as rapidly as possible a moderately pure periplasmic fraction, we did not find it necessary to eliminate this small amount of unknown contamination. Although we have not tested it directly, we presume that the procedure would also be effective with very large cultures (e.g., 200-liter volumes from a fermentor run).

How chloroform acts to release only periplasmic proteins is not known. It does not seem to act by preferentially dissolving the outer membrane, because characteristic and abundant outer membrane proteins, such as porins, did not appear in the shock fluid. The removal of a specific protein which acts as a plug in outer membrane gaps may allow the release of the entire periplasmic protein complement. It is interesting that toluene, which has been used extensively to permeabilize cells, actually antagonizes the chloroform action. We have no explanation for this effect.

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